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Effect of Acetaminophen on Oxidant and Irritant Respiratory Tract Responses to Environmental Tobacco Smoke in Female Mice

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Running title: Acetaminophen and airway oxidative stress

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Abstract

Background: Although it is known that acetaminophen causes oxidative injury in the liver; it is not known if it causes oxidative stress in the respiratory tract. If so, this widely used analgesic may potentiate the adverse effects of oxidant air pollutants.

Objectives: The goals of this study were to determine if acetaminophen induces respiratory tract oxidative stress and/or potentiates the oxidative stress and irritant responses to an inhaled oxidant: environmental tobacco smoke (ETS).

Methods: Female C57Bl/6J mice were administered acetaminophen (100 mg/kg ip) and/or side stream tobacco smoke (as a surrogate for ETS, 5 mg/m^3 for 10 minutes) with airway oxidative stress being assessed by loss of tissue antioxidants (as estimated by non-protein sulfhydryl, NPSH, levels) and/or induction of oxidant stress response genes. In addition, the effects of acetaminophen on airway irritation reflex responses to ETS were examined by plethysmography.

Results: Acetaminophen diminished NPSH in nasal, thoracic extrapulmonary, and lung tissues, and also induced the oxidant stress response genes, glutathione cysteine ligase, catalytic subunit, and NAD(P)H dehydrogenase, quinone 1, in these sites. ETS produced a similar response. The response to acetaminophen plus ETS was equal to or greater than the sum of the responses to either agent alone. Although without effect by itself, acetaminophen greatly increased the reflex irritant response to ETS.

Conclusions: Acetaminophen, at supratherapeutic levels, induced oxidative stress throughout the respiratory tract and appeared to potentiate some responses to environmentally relevant ETS exposure in female C57Bl/6J mice. These results highlight the potential for this widely used drug to modulate the responsiveness to oxidant air pollutants.

Introduction

Oxidative stress results from an imbalance between antioxidants and pro-oxidants within a cell. Oxidative stress is a common mechanism for respiratory tract injury by inhaled and also systemically delivered toxic agents. For example, oxidative stress contributes to the airway injury produced by inhaled diesel exhaust, ozone, and environmental tobacco smoke (ETS) (Holguin 2013). Systemically delivered toxicants such as styrene and naphthalene can be bioactivated throughout the respiratory tract, and induce oxidative stress as well (Cruzan et al. 2012; Spiess et al. 2010). Oxidative stress contributes to the development and/or exacerbation of respiratory diseases, including asthma. For example, biomarkers of oxidative stress are elevated in asthma and individuals with low antioxidant levels are susceptible to the development of this disease (Larkin et al. 2015; Riedl and Nel 2008). Additionally, oxidant air pollutants, including the ubiquitous indoor air pollutant ETS, are associated with increased prevalence and/or severity of asthma (Gilmour et al. 2006; IOM 2000, Kanchongkittiphon et al. 2015). This report focuses on the potential for the over-the-counter analgesic, acetaminophen, to induce airway oxidative stress and potentiate the airway response to the inhaled oxidant stressor, ETS.

Acetaminophen, (APAP, *n*-acetyl-*para*-aminophenol), is a commonly used medicine for relieving pain and reducing fever in adults and children. APAP is a known hepatotoxicant. The majority of APAP is metabolized in the liver by glucuronidation and sulfation pathways and is safely excreted; however, a fraction of APAP is metabolically activated in the liver to the pro-oxidant metabolite N-acetyl-P-benzoquinone-imine (NAPQI) (McGill and Jaeschke 2013). NAPQI is highly reactive, causes cellular oxidative stress, and covalently binds to cellular macromolecules (Jaeschke et al. 2012). Detoxification of NAPQI consumes the important antioxidant glutathione (GSH). NAPQI induces the nuclear factor-erythroid 2-related factor-2

(NRF2) dependent oxidative stress gene response pathway (Bataille and Manautou 2012; Klaassen and Reisman 2010) causing induction of genes involved in multiple detoxification pathways. This includes the enzyme which catalyzes the rate-limiting step of GSH synthesis, glutamyl-cysteine ligase, catalytic subunit (GCLC), as well as NAD(P)H dehydrogenase, quinone 1 (NQO1), which is involved in the detoxification of reactive quinones (Aleksunes et al. 2006; Chan and Kwong 2000). Induction of NRF2 pathway genes can be used as a sensitive biomarker for cellular oxidative stress (Cichocki et al. 2014a, b; Klassen and Reisman 2010). Specifically, in the typical hierarchical response pattern to oxidants, induction of NRF2 genes occurs at doses lower than those needed to induce inflammation or cause cytotoxicity (Nel et al., 2006).

The metabolic activation of APAP to NAPQI is catalyzed by a variety of cytochrome P450 isoforms including CYP2E1, CYP3A4, and CYP1A2 (Hinson et al. 2010). These CYPs are expressed in the respiratory tract, suggesting that similar metabolic activation may occur in this site as well (Ding and Kaminsky 2003). When administered directly to the lungs via intratracheal instillation, NAPQI induces a neurogenic inflammatory response (Nassini et al. 2010) and hepatotoxic doses of acetaminophen are known to deplete GSH and cause injury in the nose and lungs when administered systemically (Hart et al. 1995; Gu et al. 2005). It is not known if the bioactivation capacity of respiratory tissues is sufficient to induce oxidative stress at non-hepatotoxic, supratherapeutic doses of APAP. Were APAP to induce oxidative stress in respiratory tissues, it could enhance the response to other oxidant stressors. The current study is focused on determining if APAP induces oxidative stress in respiratory tissues, and if so, if APAP enhances the oxidative stress and respiratory tract irritant responses to environmentally relevant ETS exposure. ETS was selected because it is a common air pollutant and both

acetaminophen and ETS have been associated with increased prevalence of asthma (Etminan et al. 2009; Gilmour et al. 2006; IOM 2000; McBride 2010).

The hypothesis that APAP acts as a pro-oxidant in the airways and enhances the response to ETS was tested in a mouse model by determining if APAP and/or APAP+ETS: 1) causes a loss of GSH, and 2) activates the oxidant stress response pathway, as indicated by the activation of two NRF2-dependent genes: *Gclc* and *Nqo1*. Because the intent was to determine if the dosages of APAP or ETS were sufficient to alter normal homeostatic levels of NPSH or gene expression, the data for these parameters were expressed as percent of control. Precise quantitative comparisons between treatment groups were made within individual experiments (which shared the same control group). Only generalized comparisons were made across experiments where control levels may have differed. The effects of APAP on the response to ETS were further characterized by examining the ETS-induced irritation reflex response. This response is caused by ETS stimulation of nasal trigeminal chemosensory nerves through the oxidant sensitive transient receptor potential ankyrin 1 (TRPA1) channel (Andre et al. 2008).

Materials and Methods

Experimental Approaches. The first studies were aimed at determining if APAP induced oxidative stress in respiratory tissues. Towards this end, animals were euthanized 0-3 hrs after APAP administration (ip), and nasal respiratory/transitional mucosa (RTM), intrathoracic airways (tracheal/mainstream bronchial mucosa, TBM), lung (left lobe), and liver samples were collected. For euthanasia, mice were anesthetized with urethane (1.3 g/kg) followed by exsanguination in the laboratory between 10:00 am and 12:00 am. Oxidative stress was assessed by determination of tissue non-protein sulfhydryl levels (NPSH, as a surrogate for GSH), and expression levels of two NRF2-dependent oxidant stress response genes: *Gclc* and *Nqo1*

(Cichocki et al. 2014b). The response of NRF2 -/- mice to APAP was also investigated to confirm a role for NRF2 in any gene expression changes. Plasma levels of acetaminophen were determined in animals euthanized 15 min after 100 mg/kg dosing (the expected time of peak plasma concentration based on previous studies, Gu et al. 2005; Lin et al. 1996), to assess the therapeutic relevancy of the APAP dosing regimen.

The interaction between APAP and ETS was assessed by examining the nasal RTM response of C57Bl/6J wild-type mice (tissue NPSH levels, and *Gclc* and *Nqo1* expression) to these agents alone and in combination. Side-stream smoke was used as a surrogate for ETS. Mice were exposed for 10 min to a nominal exposure concentration of 5 mg/m^3 to approximate the ETS concentrations achieved in a closed automobile containing a smoker, or clean filtered air in the same apparatus (Sendzik et al. 2009). The 10 min duration corresponded to the burn time of a single cigarette. In addition, the effect of APAP on the reflex irritation response of mice to ETS was examined. Stimulation of trigeminal chemosensory nerves causes a brainstem-mediated characteristic change in breathing pattern that can be assessed non-invasively during exposure as described in detail below (Alarie 1973; Vijayaraghavan et al. 1993; Willis et al. 2011). Stimulation of these nerves is also pro-inflammatory (Andre et al. 2008; Caceres et al. 2009; Nassini et al. 2010). The rationale for the focus on the nose for the APAP-ETS study was multifold. The nose is the first airway exposed to ETS in the mouse, and is a common site of inhaled toxicant-induced injury in the rodent (Morris 2012). Nasal injury in nose-breathing rodents is a sentinel for lower airway injury in mouth breathing humans (Morris, 2012; Cichocki et al. 2014b), and the ETS-induced irritant reflex response is mediated via the trigeminal nerve and, therefore, originates in the nose (Gloede et al. 2011; Morris 2012).

The role of oxidative stress in inducing the irritant reflex response was examined by multiple approaches. First, the effects of APAP on the responses to the pro-oxidant irritant acrolein, and the non-oxidant irritant cyclohexanone were examined to confirm any effects of APAP were oxidant specific rather than generalized in nature. Cyclohexanone activates chemosensory nerves by the transient receptor potential vanillin 1 (TRPV1) receptor, while acrolein acts through TRPA1 (Bautista et al. 2006; Saunders et al. 2013). Second, the effect of APAP was examined in animals pretreated with 5-phenyl-1-pentyne (5-PP) to inhibit CYP metabolism (Morris 2013; Roberts et al. 1998) and bioactivation of APAP. Third, the GSH depleting agent, diethyl maleate (DEM), was administered to determine if modulation of nasal GSH status could replicate the effects of APAP. This agent is conjugated with GSH via glutathione-S-transferases, resulting in decreased tissue GSH levels (Boyland and Chasseaud 1967; Phimister et al. 2004).

Animal procedures. Female C57Bl/6J mice (9 to 11 weeks of age) were used for all experiments. Female mice were used because there is a rich database on respiratory reflex responses to irritants, including ETS, in female mice (Willis et al., 2011; Ha et al., 2015) and female mice are more sensitive than male mice to the acute respiratory tract effects of metabolically activated toxicants (Van Winkle et al., 2002). Mice were obtained from Jackson Laboratories. Age-matched Nrf2-null and wild-type (C57BL/6J background) mice were kindly provided by Dr. Jose Manautou. Initial Nrf2 -/- breeding pairs were obtained from Dr. Angela Slitt at the University of Rhode Island. Mice were housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities at the University of Connecticut under standard environmental conditions (12-h light-dark cycle at 23°C). Mice were housed over hardwood shavings in groups of 5 mice per cage (Sani-Chip Dry, P. J. Murphy Forest Products). Food (Lab Diet; PMI Nutrition International and tap water were provided *ad libitum*. A total of

570 mice were used for these studies. All animals were treated humanely and with regard for alleviation of suffering. Animal procedures were approved by the University of Connecticut Institutional Animal Care and Use Committee.

Unless otherwise indicated all chemicals were obtained from Sigma Aldrich . APAP, dissolved in 37^{o} C saline (10 mg/ml), was administered via ip injection at doses of 60,100 or 200 mg/kg. When administered, the cytochrome P450 inhibitor 5-PP (GFS chemicals) was given ip at a dose of 100 mg/kg (10 mg/ml in olive oil) 1hr prior to APAP treatment (Morris 2013). DEM was administered at a dose of 250 mg/kg (0.33 M solution in olive oil, ip, Phimister et al. 2005). Control animals received vehicle injections. Mice were exposed to airborne irritants as described below; irritant exposure concentrations were selected to produce demonstrable, but submaximal irritation. Nasal RTM tissues were removed from the ventral portions of the nasal cavity by microdissection (Cichocki et al. 2014a) (Olfactory mucosa was not collected as this is neural, non-respiratory, tissue.) The intrathoracic TBM airways and the left lobe of the lungs were removed. For NPSH determination tissue samples were homogenized in 5% trichloroacetic acid-3 mM EDTA and were stored at -80 ^{o}C until analysis. For qRT-PCR, tissues were immediately placed in an aqueous RNA stabilization buffer containing saturating ammonium sulfate, 20 mM EDTA, and 25 mM Sodium Citrate, at pH 5.2 and stored at -80 ^{o}C until analysis.

Breathing Pattern Analysis. Mice were held in a double plethysmograph (Buxco Inc.) connected to a directed airflow nose-only inhalation chamber (CH Technologies) for irritant exposure to allow monitoring of breathing parameters during the exposure. Animals were placed in the plethysmograph for a 15-min acclimatization, a 5-min baseline, and then a 10 minute exposure to irritant. The sensory irritation reflex response, characterized by a pause (termed braking) at the onset of each expiration due to glottal closure, was quantitated by measuring the

duration of braking (Willis et al., 2011). Breathing patterns were monitored using a Buxco mouse pneumotachograph with a Buxco pressure transducer coupled to automated Emka Technologies Iox 2 software. This software automatically measured the duration of braking for each breath and then averaged those data over each minute (typically 150-300 breaths) to provide minute-by-minute averages over a 30-min duration of measurement. Plethysmographic-based assessment of breathing patterns based on 1 minute averages is the long-accepted approach for assessment of sensory irritation (Alarie, 1981; Vijayaraghavan et al. 1993; Morris et. al. 2003).

Respiratory Irritant Exposures. Mice were exposed to ETS for 10 minutes or to the irritant vapors, acrolein or cyclohexanone, for the same duration. Control mice were exposed to clean filtered air in the same exposure chamber. Mice were continuously exposed to constant levels of irritant to allow for the most precise estimation of irritant- or APAP- induced changes in breathing. For exposure clean- or irritant-laden air was drawn into the headspace of the double plethysmograph at a flow rate of 1L/min.

Acrolein (nominal concentration 2.5 ppm) atmospheres were generated by flash evaporation; cyclohexanone (nominal concentration, 1500 ppm) atmospheres were generated by passing filtered air through liquid cyclohexanone in a gas washing bottle; airborne vapor concentrations were monitored by gas chromatography using a Varian 3800 gas chromatograph as described previously (Willis et al. 2011). To achieve constant concentration smoke exposures, side stream cigarette smoke was continuously generated by passing filtered and humidified air over a lit cigarette centered in one port of a 2L four neck boiling flask (Kimble-Chase). High concentration smoke from the flask was drawn into a second identical flask using a peristaltic pump and fed using positive pressure into the inhalation chamber. Smoke was generated from Kentucky 2R4F reference cigarettes that had been stored for at least 24 hr at 55% relative

humidity. For smoke exposures, particulate levels were measured by a Casella Microdust Pro Analyzer. Nominal suspended particle concentration over the duration of smoke exposures was 5 mg/m^3. Airborne carbon monoxide (CO) levels were monitored continuously during exposure with a Digital CO Detector (DCO1001, General Tools). CO levels averaged 25 to 35 ppm throughout the exposures.

Analytical Techniques. NPSH levels were used as a surrogate for GSH. NPSH was determined colorimetrically in a 96-well plate assay using a reduced GSH standard curve based on the method of Sedlak and Lindsay (1968). NPSH data were normalized to protein content using a colorimetric 96-well plate assay using a BSA standard curve based on the Lowry method (Lowry et al. 1951). NPSH data are expressed as percent of control. Control NPSH levels were determined empirically and the data for exposed groups expressed as the average value among concurrent controls Overall, control values averaged 18, 8, and 12 nmol/mg protein in RTM, TBM and lung, respectively. For determination of plasma APAP levels, blood was collected by cardiac puncture with heparinized syringes from mice euthanized 15 minutes following a 100 mg/kg dose of APAP. Plasma, prepared by centrifugation, was diluted 1:10 in 5% trichloroacetic acid-3 mM EDTA and then analyzed for APAP content by HPLC based on the method of Lin et al. (1996). APAP eluted with a retention time of 11 minutes using a mobile phase of 7% acetonitrile-0.1% trichloroacetic acid, a flow rate of 1 mL/min, and an Agilent Eclipse-Pro Plus LC-18 (5µm, 4.6 x 250 mm) column, and was detected by UV absorbance at a wavelength of 254 nm. Levels were determined in plasma from 4 APAP-injected and 4-vehicle injected mice (to confirm the absence of contaminating peaks).

For qRT-PCR of selected oxidant stress response genes, total RNA was isolated from respiratory tissue homogenates using an RNeasy kit from Qiagen. 1 µg of total RNA was used for first

strand cDNA synthesis by an iScript cDNA synthesis kit (Bio-rad). RT-PCR was performed using SYBR Green as an indicator with an ABI 7500 Real-Time PCR system on the Fast setting. PCR reactions contained 10 ng of cDNA (4µl), 500 nM of each primer (1µl total), and 5 µl of 2x SYBR Green PCR Master mix for a 10µl total volume. The PCR was carried out according to the manufacturer's recommended thermal cycling protocol. Data were normalized to β-Actin as the internal reference control mRNA. Results are represented as the fold change in expression of target genes over control calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Primers were designed with Life Technologies OligoPerfect™ designer and obtained from Invitrogen (Life Technologies). See Table 1 for list of primer sequences.

Statistical Analysis. Numbers of animals per group were selected to detect a 25% difference between groups based on our previous experience with the methodologies with α=0.05 at 80% power. Data were analyzed by XLSTAT (Addinsoft). Individual data values were excluded *a priori* if they deviated from the mean by more than 3 standard deviations. (Of the approximately 600 mice used in this study, data from 5 mice were excluded due to this exclusion criteria.) Data are reported as mean ± SE unless otherwise indicated. Data were compared by ANOVA followed, as appropriate, by Newman-Keuls test. When appropriate data were $\log_{10}$ transformed to correct for heteroscedasticity. Sensory irritation time course data were analyzed by repeated-measures ANOVA followed by Newman-Keuls test. A p-value less than 0.05 was required for statistical significance.

Results

Airway oxidative stress responses to APAP. To examine the time course of the response to APAP mice were euthanized 1, 2 or 3 hrs after 100 mg/kg APAP treatment and tissues collected from RTM, TBM and lung. In all tissues, NPSH levels were approximately 80% of control 1 hr

after APAP ($p<0.05$) and returned to control levels by 2-3 hrs (Figure 1a). At a dose of 60 mg/kg RTM NPSH was not diminished by APAP, averaging 96 ± 7.7 % of control. Liver NPSH levels averaged 60 ± 3.9%, 83 ± 2.7%, and 106 ± 3.5%, at 1, 2 and 3 hr, respectively after a 100 mg/kg dose of APAP. Both *Gclc* and *Nqo1* genes were significantly induced in all respiratory tissues at a dose of 100 mg/kg, albeit with somewhat differing magnitudes and time courses (Figure 1b, c). Dose response relationships for RTM gene induction are shown in Figure 1d. While *Gclc* was significantly induced at a dose of 60 mg/kg APAP, *Nqo1* was not. Both *Gclc* and *Nqo1* were significantly induced at a dose of 100 mg/kg APAP. In NRF2 -/- mice, basal RTM expression of *Gclc* and *Nqo1* were approximately 17% and 2% of wild-type control, respectively (7- and 70-fold lower than wild-type control) (Figure 1e). In APAP treated mice *Gclc* averaged 33% of wild type-control and *Nqo1* averaged 4% of control. Thus 2-fold or less induction of either gene was observed. The gene levels in APAP treated knockout mice did not differ from control knockout mice. Serum APAP levels were determined in mice euthanized 15 min after a 100 mg/kg dose. No APAP (or contaminating peak) was detected in vehicle-injected controls (n=4), APAP levels averaged 35 ± 6 μg/mL (n=4) in treated mice.

APAP-ETS interaction. Initial studies focused on the time course of NRF2-dependent gene induction, if any, following ETS (Figure 2). ETS exposure levels averaged 6.3 ± 0.6 mg/m^3 (mean ± SD). *Gclc* was slightly induced, with *Gclc* levels averaging 1.1- and 1.2-fold of control at 1 and 2 hr respectively ($p<0.05$). *Nqo1* was only increased 2 hrs after exposure to 2-fold of control ($p=0.02$, t-test).

To examine the potential for an APAP-ETS interaction, mice were exposed to ETS 1 hr after 100 mg/kg APAP administration because this is a time when there is a significant decrease of NPSH (20%) but no induction of antioxidant genes in the RTM (see Figure 1). The dose of 100 mg/kg

was selected because at this dose APAP affected all measures of oxidative stress (see Figure 1). Mice were euthanized immediately after ETS exposure for determination of NPSH (Figure 3a) and 2 hours after ETS (3 hours after APAP) for assessment of gene expression because both *Gclc* and *Nqo1* were induced at that time by ETS (Figure 2). It can be noted that *Gclc*, but not *Nqo1*, was induced by APAP at this time point (Figure 1).

Exposures to ETS alone and APAP alone were associated with non-significant decreases in NPSH (20% and 19%, respectively), while exposure to APAP followed by ETS caused a 40% reduction ($p=0.04$, compared to control, Figure 3a). NPSH was significantly diminished from control in only the APAP+ETS groups. The NPSH levels (81%) in APAP treated mice in this study were not different from controls. In contrast NPSH levels of 80% of control in RTM 1 hr after exposure to 100 mg/kg APAP was significantly lower than controls (Figure 1A). (In both studies NPSH was measured 1 hr after APAP administration.). ETS exposure levels averaged 5.2 ± 0.4 mg/m^3 (mean ± SD).

Gclc was significantly increased over control levels in the APAP and the APAP+ETS groups (Figure 3b). In the ETS group, *Gclc* expression levels averaged 1.2-fold of control, similar to that in the previous experiment (Figure 2a), but in this case a significant difference from control was not observed. In the APAP and APAP-ETS groups, *Gclc* expression averaged 2.8 and 3.7-fold of control suggesting an additive or greater interaction. The difference in *Gclc* levels in the APAP and APAP+ETS groups approached statistical significance ($p=0.07$).

Nqo1 was significantly increased over control in only the APAP+ETS group (Figure 3c) and the levels in the group were significantly higher than in either the APAP or ETS groups. In the ETS group, *Nqo1* levels averaged 1.3-fold of control levels, a response somewhat less than in the

previous experiment (Figure 3b); direct comparison of the response level in this and the preceding experiment did not reveal a significant difference. *Nqo1* was decreased in the APAP group, but not significantly compared to controls ($p > 0.9$), consistent with the lack of expression changes for *Nqo1* in RTM 3 hr after APAP in the earlier experiment (see Figure 1c). ETS exposure levels averaged 4.7 ± 0.5 mg/m^3 (mean ± SD).

Irritation Reflex. ETS induces the sensory irritation reflex response as indicated by induction of braking at the onset of each expiration. This is shown by the period of no airflow (horizontal line) in each breath in (Figure 4a); no such response was observed after APAP or saline vehicle (Figures 4b, c), but an enhanced response was seen in APAP+ETS mice (Figure 4d). This response was quantitated by calculating the 1 min average duration of braking in each animal (Figure 4e). While APAP alone did not elicit this response, ETS (4.5 ± 0.6 mg/m^3, mean ± SD) produced a moderate response which was significantly increased in APAP-pretreated mice, indicating that APAP potentiated the irritation response.

To examine the oxidant basis for this interaction, the effects of APAP on the irritation response to the TRPA1 specific oxidant vapor, acrolein, and the non-oxidant, TRPV1 agonist vapor, cyclohexanone were examined (Willis et al. 2011) (Figures 5a,b). APAP was without effect on the response to cyclohexanone, but potentiated the response to acrolein. To establish a dose-response, two additional dose groups (60, and 200 mg/kg) were included in the acrolein sensory irritation experiment. The response to acrolein was slightly but not significantly ($p=0.6$) elevated by a 60 mg/kg dose of APAP (Figure 5a) and significantly ($p=0.001$) elevated at a dose of 100 mg/kg. At 200 mg/kg APAP produced braking during the baseline (data not shown). Additional studies were done to further characterize the role of oxidative stress in the APAP potentiation of the irritant response (Figure 5c). These studies focused on the APAP potentiation of the acrolein

irritant response, rather than ETS, because acrolein is a single agent known to act through TRPA1 (Willis et al. 2011). As observed previously, the irritation response to acrolein was potentiated by APAP. The potentiation was absent in 5-PP-treated mice. Pretreatment with DEM 1 hr prior to acrolein exposure potentiated the acrolein response. Nasal RTM NPSH levels in the DEM treated mice averaged 75 ± 5.4% percent of control (compared to 100 ± 6.6% in controls, p=0.04, n=4 in each group). This level is similar to that caused by APAP (Figure 1a). 5-PP was without effect on the irritant response to acrolein (in mice not given APAP), and the DEM vehicle was without effect on the response to acrolein (data not shown). In fact, the durations of braking were within 50 msec of each other and did not differ significantly among any of the control groups (non-treated, DEM vehicle-treated and 5-PP-treated), therefore, for the sake of simplicity these groups were all pooled to form the composite control group for this study.

Discussion

The present study demonstrates that APAP, at supratherapeutic doses, modulates airway oxidative stress- and respiratory irritant-responses to ETS. That adverse respiratory responses to ETS can be enhanced by the commonly used analgesic APAP, is a novel concept. Greater than 97% of children use acetaminophen at recommended doses of 15 mg/kg or less prior to age two (Kogan et al. 1994). Historically, ETS exposure in non-smoking populations has been high. During the period from 1988-1991, greater than 80% of non-smoking adults in the U.S. were passively exposed to ETS (Pirkle et al. 1996). More recently, ETS exposure in non-smokers has ranged from 52.5% in 1999 to 25.3% in 2012 (CDC 2015). Approximately 30% of children are exposed to ETS in an automobile (King et al. 2012). The ubiquitous use of APAP, coupled with

the high frequency of exposure to ETS, highlights the widespread potential for adverse health effects were a toxicologically significant interaction between APAP and ETS to occur.

Our initial studies indicated that a 60 mg/kg dose of APAP did not cause a significant changes in measures of oxidative stress: nasal GSH was not significantly decreased, *Gclc* expression was significantly higher than control, but *Nqo1* expression was not. Additionally, 60 mg/kg caused a slight increase in the acrolein response but the increase was not statistically significant. APAP doses of 100 mg/kg were used for subsequent studies because this was the lowest dose which produced significant changes in all parameters. Future studies could more thoroughly define the effects of APAP at lower doses. Interestingly, at 200 mg/kg, APAP produces a sensory irritation response suggesting there may be sufficient electrophile produced at this dose to interact with airway sensory nerves. This finding is consistent with the observation that NAPQI can directly activate the TRPA1 channel (Nassini et al. 2010). The human recommended dose of APAP is 15 mg/kg. Therapeutic levels are 5-20 μg/ml and hepatoxicity is associated with blood levels exceeding 150 μg/ml (Rumack and Matthew 1975) in adults. Although the dose level used in this study is higher than the recommended dosage, peak blood levels were 35 μg/ml in the adult mice used in this study. This is similar to that reported by Gu et al. (2005), who also report that blood APAP decreases to 10 μg/ml within 1 hour after a 100 mg/kg dose. Thus, this dosing regimen resulted in near therapeutic APAP levels that were well below the threshold for overt liver toxicity. The ip route of administration in this study may have led to higher peak APAP levels that would be observed after oral administration because absorption is slower following oral than ip administration; liver first pass phenomenon would occur following either dosage regimen.

Female mice were used for these studies. Female mice are less susceptible to APAP hepatotoxicity than males (Sheng et al 2013), but more sensitive to the pulmonary toxicity of metabolically activated chemicals such as naphthalene (Van Winkle et al. 2002). Future studies would be needed to determine if sensitivity to the pulmonary effects of APAP and ETS differs between male and female mice.

At the 100 mg/kg dose APAP clearly induced oxidative stress in the airways as indicated by a decrease in NPSH and induction of oxidant stress response genes. It has previously been shown that APAP depletes nasal NPSH (Gu et al. 2005), but at much higher, hepatotoxic doses. That Nrf2 -/- mice demonstrated reduced responsiveness to APAP provides evidence that the oxidant stress gene response was mediated, at least in part, through this transcription factor. The toxic response to APAP is due to metabolic activation via CYP to the strong electrophile NAPQI and CYP is expressed throughout the respiratory tract of the mouse (Ding and Kaminsky 2003; Hinson et al. 2010). Perhaps local activation of APAP is involved in the responses observed herein. The current results do not, however, rule out the possibility of hepatic events (escape of activated APAP, decrease of blood GSH) as a contributing factor to the oxidative stress response observed in this study (Gu et al. 2005; Phimister et al. 2004). Since, 5-PP inhibits CYP metabolism systemically these data do not provide insights into the exact role of hepatic versus local nasal activation of APAP.

ETS contains thousands of chemicals, many of which are oxidants (Gilmour et al. 2006). Exposure to e to 5 mg/m^3 ETS for 10 minutes caused a modest oxidative stress response as indicated by slight induction of an oxidative stress gene response. While responses of similar magnitude were observed in the first ETS study (Figure 2) and the ETS-APAP study (Figure 3), responses were significantly different from controls in only one experiment. This suggests the

ETS exposure represents a threshold response level because the magnitude of response was quite low and the response was significantly different from control in only one of two experiments. If so, this may represent a response of concern because the exposure level is similar to that obtained in a car containing an active smoker (Sendzik et al. 2009) and the exposure was only of 10 min duration. Future studies would be required to determine if slightly higher concentrations or longer duration ETS exposure results in a repeatedly observed oxidative stress response.

The interaction between APAP and ETS is likely due to the oxidant properties of both agents. The NPSH loss response of both agents appeared to be additive based on the 40% decrease in NPSH observed in the APAP+ETS groups compared to the 20% decreased observed in the APAP alone and ETS alone groups, although a formal statistical test to confirm this was not done. The induction of *Gclc* also appeared to be additive, although the differences between APAP and APAP + ETS did not quite attain statistical significance. The *Nqo1* response clearly indicated a synergistic interaction between APAP and ETS, with neither agent alone producing a response, but the combination causing a clear induction. A synergistic interaction was also observed with respect to the reflex irritation response to ETS. APAP alone did not cause reflex irritation, but it markedly increased the irritation response to ETS.

The finding that APAP enhances the irritation response to ETS in these studies suggests that APAP can alter complex integrated airway responses. ETS stimulates chemosensory nerves via the oxidant sensitive TRPA1 receptor (Andre et al. 2008). The current studies confirm an oxidant basis for the APAP-ETS interaction. APAP potentiated the irritant response to the oxidant acrolein, which is a known TRPA1 agonist. That APAP was without effect on the non-oxidant TRPV1 agonist, cyclohexanone, suggests the effects of APAP are oxidant specific. Treatment with 5-PP blocked the modulation of acrolein irritation indicating that oxidant induced

sensory irritation is due to the generation of a metabolite, likely NAPQI, rather than a pharmacological effect of the parent APAP. APAP is known to be activated by nasal CYP and the nasal toxicity of APAP is independent of liver activation (Gu et al. 2005). DEM at a dose which produced a similar level of GSH loss to APAP, mimicked the effects of APAP on acrolein irritation suggesting that GSH loss may contribute to the interaction. DEM has been shown to potentiate the nasal toxicity of another metabolically activated toxicant, naphthalene (Phimister et al. 2004). Perhaps nasal GSH acts to detoxify acrolein and loss of GSH enhances acrolein penetration to chemosensory nerve endings which innervate the nasal mucosa. Overall these findings provide evidence of an oxidant basis for the effect of APAP on respiratory responses, and also suggest that tissue antioxidant levels may be a determinant of sensitivity to inhaled irritant chemicals.

The public health impacts of ETS are well studied. ETS exposure is associated with increased asthma incidence and severity (Coogan et al. 2015; IOM 2000; Kanchongkittiphon et al. 2015). Multiple epidemiological studies, in both adults and children, reveal an association between the increased APAP use since 1980 and the increased asthma prevalence since that time (Barr et al. 2004; Beasley et al. 2011; Etminan et al. 2009). This has led to the "APAP hypothesis", specifically that acetaminophen may contribute to asthma causation, perhaps through its oxidant properties (McBride, 2011). This hypothesis is based simply on the observation that both APAP use and asthma prevalence have increased since 1980. This hypothesis, however is controversial due to the potential for confounding in epidemiological studies and lack of experimental evidence (Heintze and Petersen 2013; Holgate 2011). Moreover, any factor which has changed since 1980, such as air pollution, environmental chemical exposure, etc. may be responsible for the increase in asthma prevalence. The current studies lend credence to the APAP hypothesis, by

supporting a novel and biologically plausible mechanism whereby APAP may contribute to the development of asthma, specifically by enhancing the effects of other asthma risk factors such as ETS. However, the current studies only provide information on single short-term exposure to APAP and ETS. Future studies would be required to address the effects of repeated exposures to these agents and on potential differences in outcome if ETS exposure proceeded APAP administration. Nonetheless oxidative stress is thought to play a role in asthma pathogenesis (Holguin 2013; Reidl and Nel 2008) and the current studies indicate an oxidative stress-based interaction between environmentally relevant levels ETS and supratherapeutic levels of APAP.

Conclusions

Our findings indicate that in the mouse, APAP, at supratherapeutic levels, acts as an airway oxidant and potentiates acute airway responses to environmentally relevant levels of another airway oxidant, ETS. These results suggest that APAP may exert adverse effects on the respiratory tract, however in the absence of confirmatory evidence from human studies it is premature to suggest, even tentatively, changes in clinical practice.

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Table 1. Mouse primer sequences for qRT-PCR (listed 5'-3'):

Gene	Forward	Reverse
ActB	GCAACGAGCGGTTCCG	CCCAAGAAGGAAGGCTGGA
Nqo1	TTTAGGGTCGTCTTGGCAAC	GTCTTCTCTGAATGGGCCAG
Gclc	TTCATGATCGAAGGACACCA	CTGCACATCTACCACGCAGT

Figure Legends

Figure 1. NPSH levels and oxidant stress response gene expression in the respiratory tract following APAP administration. (A) Time course of NPSH levels in nasal respiratory/transitional mucosa (RTM), thoracic extra pulmonary airway mucosa (trachea/mainstem bronchi- TBM), and lung parenchyma (Lung) 1, 2, or 3 hours after APAP administration (100 mg/kg, ip). Data are presented as mean ± SE and are normalized to total protein (*= $p < 0.05$, compared to respective control). Groups contained 4-6 mice. (B, C) Time courses of (B) *Gclc* and (C) *Nqo1* expression, in nasal RTM, TBM, and Lung 1, 2, or 3 hours after APAP administration (100 mg/kg, i.p). Data are presented as mean fold change ± SE, and are normalized to control values (* = $p<0.05$, compared to respective control). Groups contained 4-6 mice. (D) Dose response relationships for *Gclc* and *Nqo1* expression 2 hours after APAP administration (60, 100 mg/kg, ip). (E) Effect of APAP on *Gclc* and *Nqo1* expression in NRF2 wildtype (WT) and knockout (KO) mice. Data are presented as mean fold change $\pm$ SE, and are normalized to control values (p values are indicated in the figure). Groups contained 4-6 mice.

Figure 2. Time course of oxidant stress response gene expression in nasal respiratory/transitional mucosa following 10 minute exposure to 6 mg/m^3 ETS. (A) *Gclc*, and (B) *Nqo1* expression data are presented as mean fold change ± SE, and are normalized to control values (* indicates $p<0.05$ compared to respective control, n=6 in all groups).

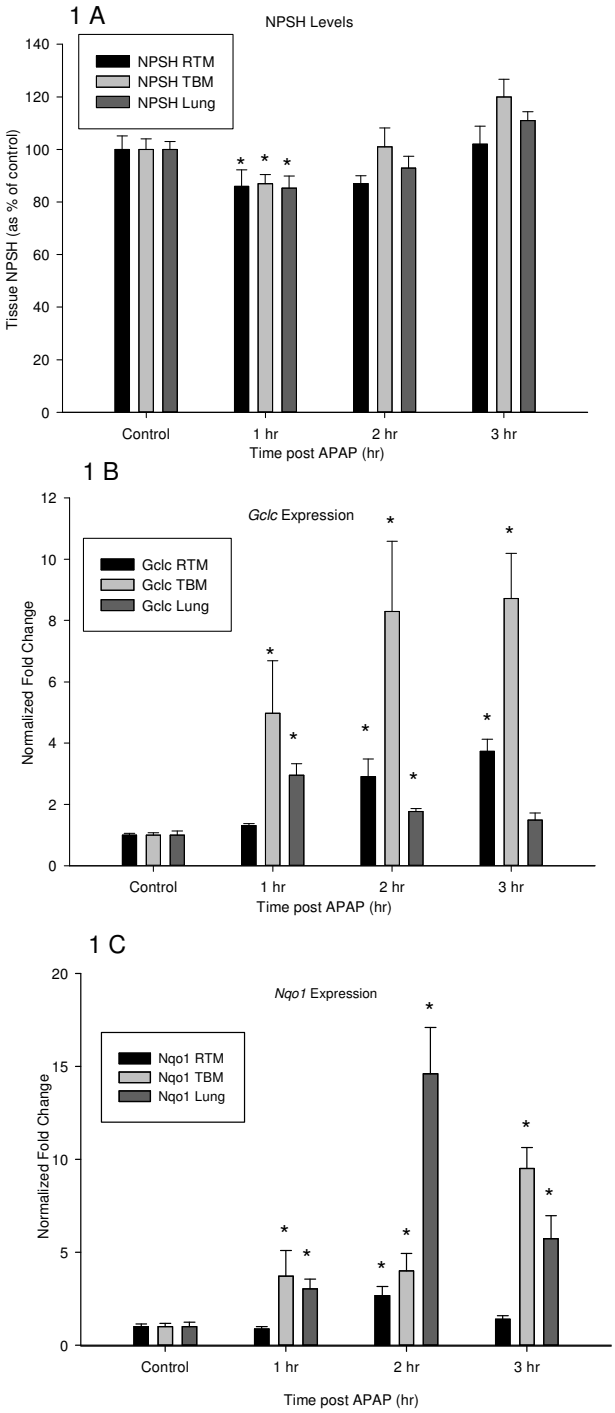
Figure 3. Effect of ETS (5 mg/m^3), APAP, and the combination of ETS+APAP on NPSH loss and oxidant stress response gene expression. Mice were exposed to ETS for 10 minutes 1 hour after APAP (or vehicle) administration. NPSH levels were determined in mice euthanized immediately after ETS exposure; Gene express was determined in mice euthanized 2 hr after ETS exposure. (A) NPSH levels in nasal respiratory/transitional mucosa in controls and after APAP, ETS, or APAP+ETS. Data are presented as mean ± SE and are normalized to total protein (*= $p < 0.05$, compared to respective control). Groups contained 5-7 mice. (B) *Gclc* and (C) *Nqo1* expression in nasal respiratory/transitional mucosa in controls and after APAP, ETS, or APAP+ETS. APAP was administered at a dose of 100 mg/kg ip; ETS exposures were of 10 minute duration at a total particulate concentration of 5 mg/m^3. *Gclc* expression in the APAP group was significantly higher than control ($p=0.001$) *Nqo1* expression in the APAP groups did

not differ from control levels (p=0.26). Data are presented as mean fold change ± SE, and are normalized to control values (* = p<0.05, compared to respective control). Groups contained 4-6 mice.

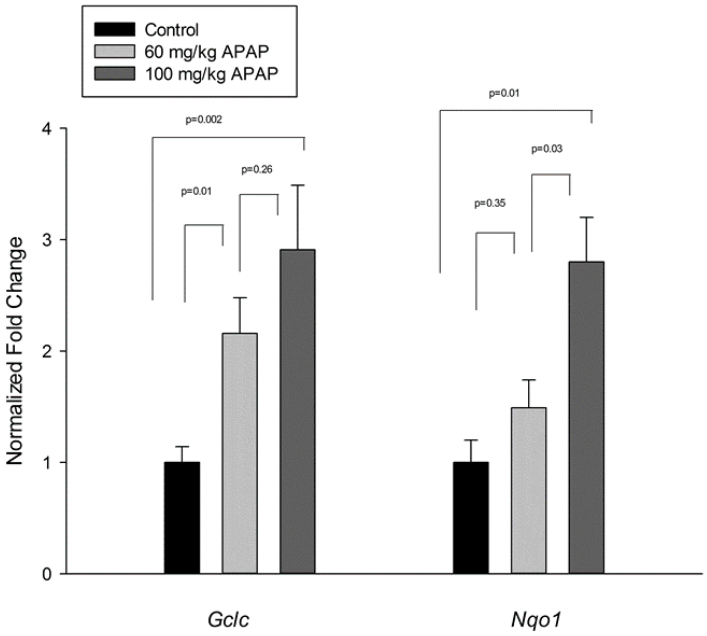
Figure 4. Representative breathing patterns of mice exposed to (A) APAP vehicle (saline) followed by 5 mg/m^3 ETS, (B) 100 mg/kg APAP followed by clean air, (C) APAP vehicle (saline) followed by clean air, (D) 100 mg/kg APAP followed by ETS. The clean air or ETS exposure occurred 1 hr following vehicle or APAP injection. Plotted are representative respiratory air flow rate patterns (arbitrary units, expiration is up) during these exposures. No braking is observed in control exposures (C) or with APAP alone (B). Marked braking, indicated by the periods of zero flow (depicted by the horizontal bars above the flow wave lines) was observed in ETS and ETS+APAP exposed mice. (E) Time course of modulation of ETS-induced reflex irritation response by APAP. Data are presented as mean duration of braking (ms) ± SE. Groups contained 5-10 mice. Data were analyzed by repeated measures ANOVA, p values shown in the figure represent the p value for the entire 10 minute exposure period.

Figure 5. Nasal trigeminal chemosensory nerve reflex irritation responses to (A) 2.3 ± 0.4 ppm acrolein (mean ± SD) following vehicle, 60 mg/kg, or 100 mg/kg APAP, and (B) 1590 ± 130 ppm cyclohexanone following vehicle or 100 mg/kg APAP administration. (C) Modulation of the nasal trigeminal chemosensory nerve irritant reflex response to 2.9 ± 0.15 ppm acrolein (control) (mean ± SD) by: APAP, 5-PP pretreatment followed by APAP, or DEM. (A, B) Time courses include a 5 min clean air baseline (-5 to 0 min) followed by irritant exposure starting at time 0 (p values are shown in figures). Data are presented as mean duration of braking (ms) ± SE. Groups contained 5-10 mice. The response to acrolein did not differ in non-treated controls, DEM vehicle treated controls or 5-PP treated controls, therefore, these data were all pooled to form the single control groups that is shown. Data are presented as mean duration of braking (ms) ± SE, Data were analyzed by repeated measures ANOVA followed by Newman-Keuls test, p values shown in the figure represent the p value for the entire 10 minute exposure period. Groups contained 4-8 mice.

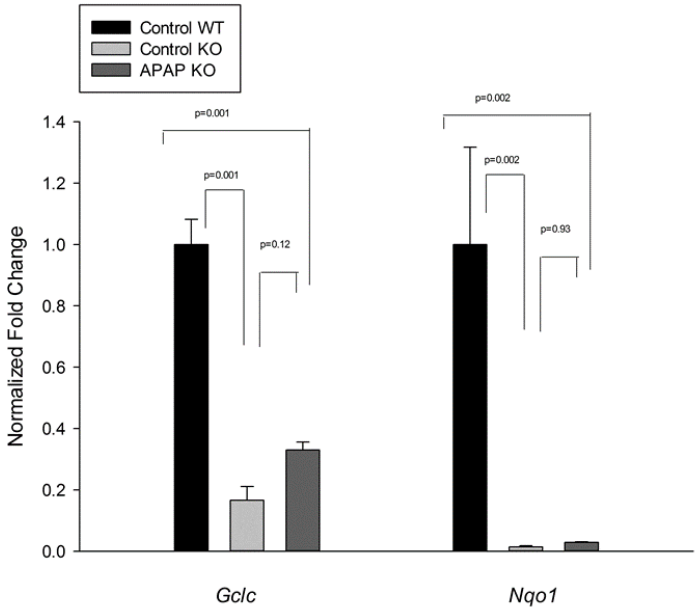
Figure 1.


1 A
NPSH Levels
NPSH RTM
NPSH TBM
NPSH Lung
Tissue NPSH (as % of control)
Control
1 hr
2 hr
3 hr
Time post APAP (hr)
1 B
Gclc Expression
Gclc RTM
Gclc TBM
Gclc Lung
Normalized Fold Change
Control
1 hr
2 hr
3 hr
Time post APAP (hr)
1 C
Nqo1 Expression
Nqo1 RTM
Nqo1 TBM
Nqo1 Lung
Normalized Fold Change
Control
1 hr
2 hr
3 hr
Time post APAP (hr)

1 D RTM Gene Expression 2 Hours After APAP

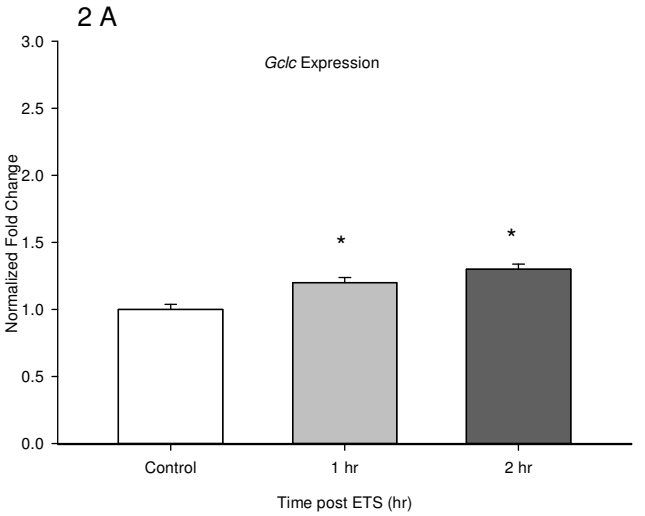


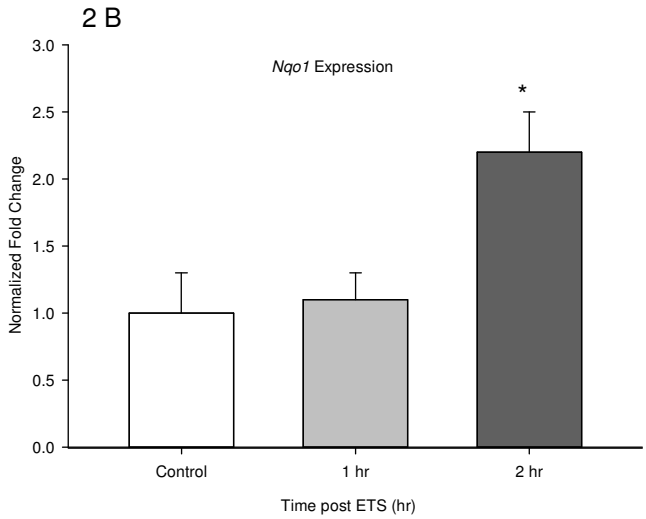
1 E RTM Gene Expression in Wild Type and NRF2 Knockout Mice



1

Figure 2.


2 A
Gclc Expression
Normalized Fold Change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
1 hr
2 hr
Time post ETS (hr)


2 B
Nqo1 Expression
Normalized Fold Change
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
1 hr
2 hr
Time post ETS (hr)

Environ Health Perspect DOI: 10.1289/ehp.1509851
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Figure 3.

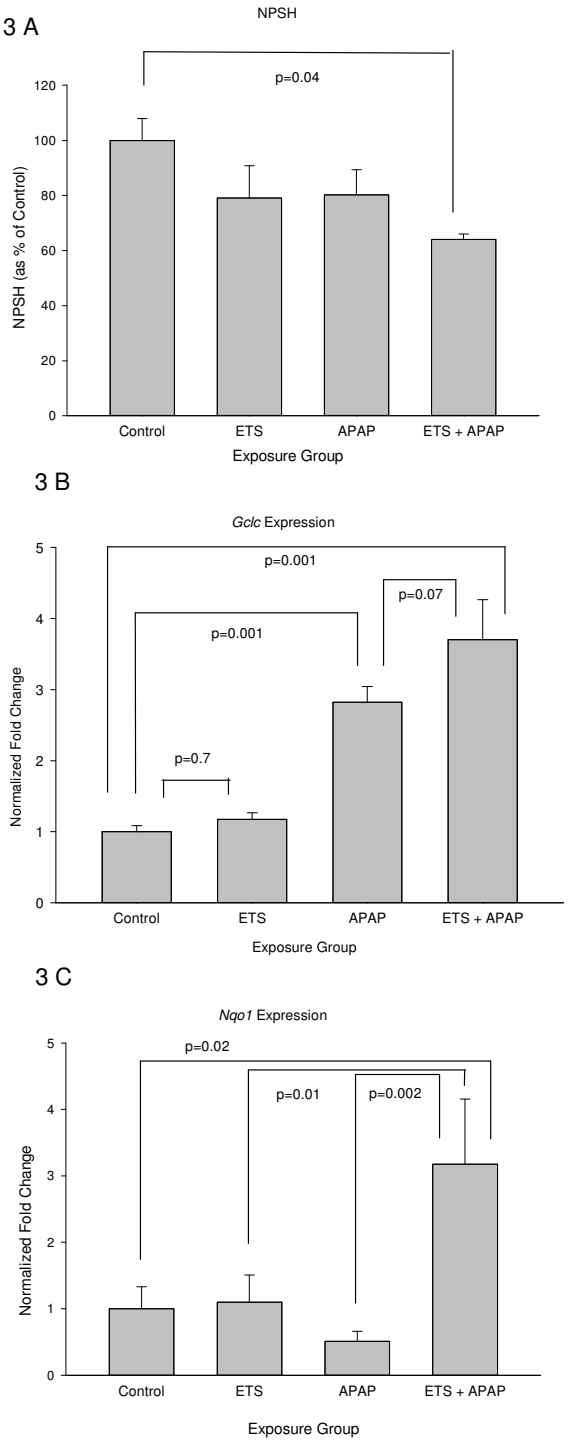


Figure 4.

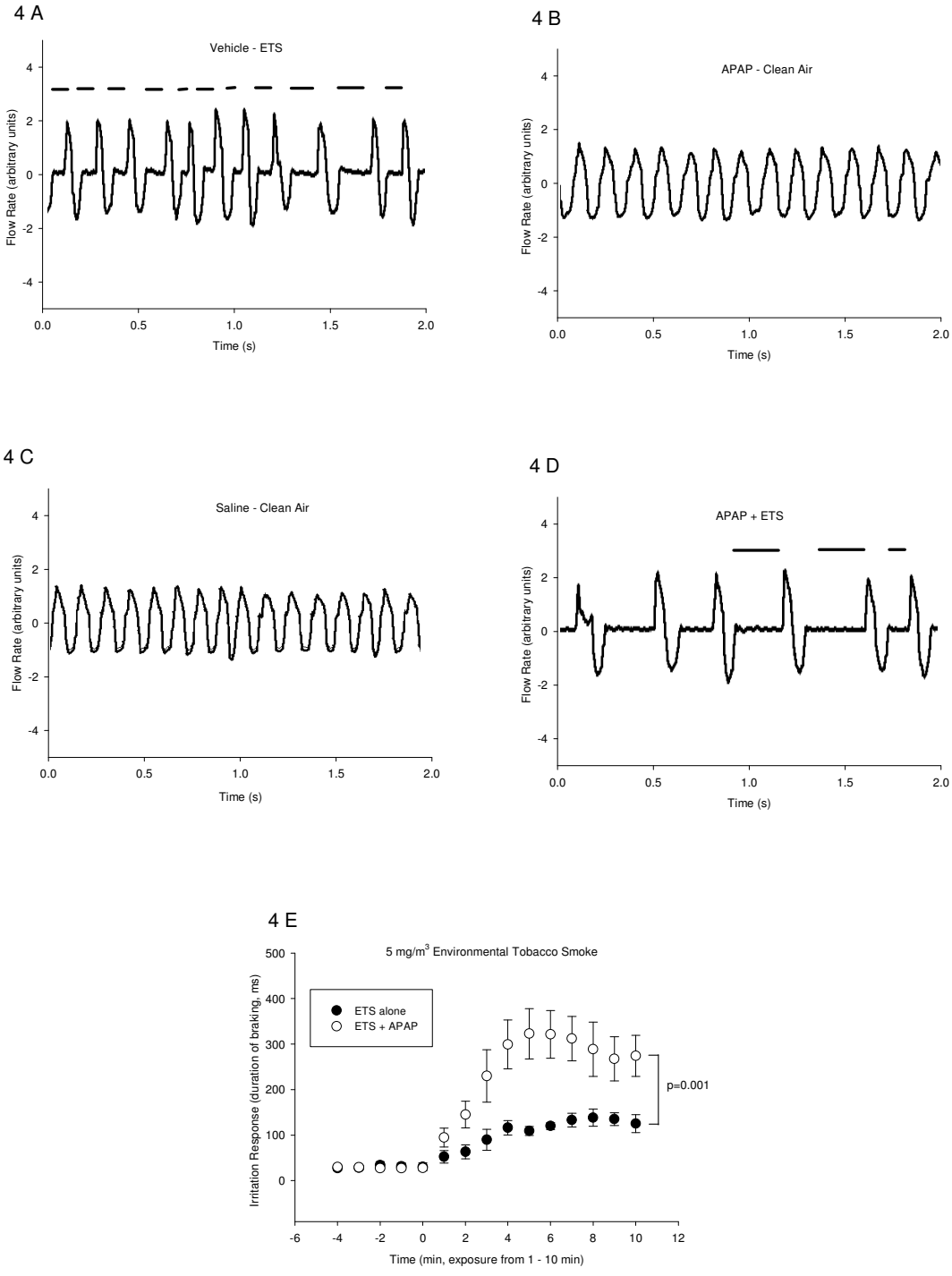
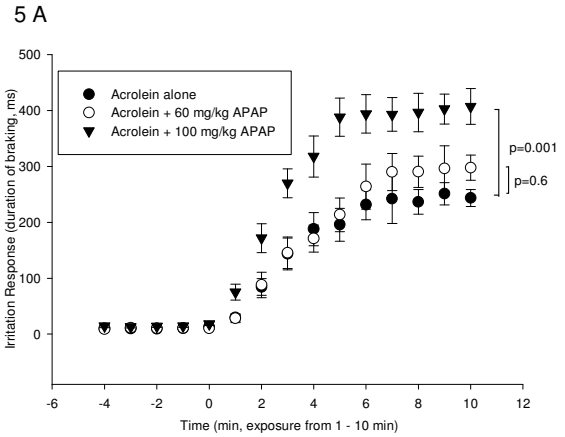
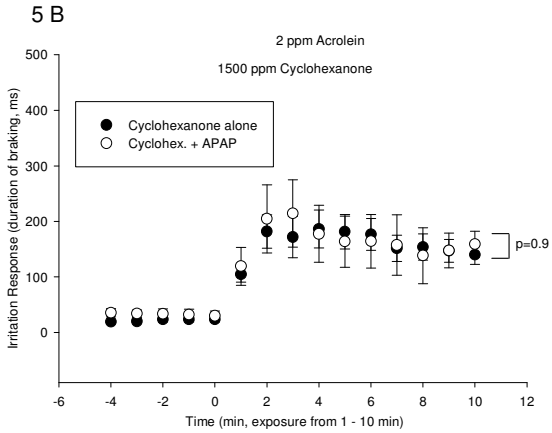
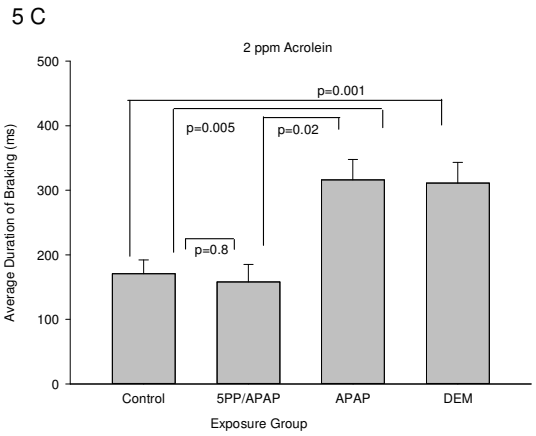

4 A
Vehicle - ETS
Flow Rate (arbitrary units)
Time (s)
4 B
APAP - Clean Air
Flow Rate (arbitrary units)
Time (s)
4 C
Saline - Clean Air
Flow Rate (arbitrary units)
Time (s)
4 D
APAP + ETS
Flow Rate (arbitrary units)
Time (s)
4 E
5 mg/m3 Environmental Tobacco Smoke
ETS alone
ETS + APAP
p=0.001
Irritation Response (duration of braking, ms)
Time (min, exposure from 1 - 10 min)

Figure 5.


5 A
Acrolein alone
Acrolein + 60 mg/kg APAP
Acrolein + 100 mg/kg APAP
p=0.001
p=0.6
Irritation Response (duration of braking, ms)
Time (min, exposure from 1 - 10 min)


5 B
2 ppm Acrolein
1500 ppm Cyclohexanone
Cyclohexanone alone
Cyclohex. + APAP
p=0.9
Irritation Response (duration of braking, ms)
Time (min, exposure from 1 - 10 min)


5 C
2 ppm Acrolein
p=0.001
p=0.005
p=0.02
p=0.8
Average Duration of Braking (ms)
Control
5PP/APAP
APAP
DEM
Exposure Group